PURIFICATION AND CHARACTERIZATION

OF

TRITIUM LABELED STARCH

by

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INTRODUCTION

Starch chemistry may be the most highly developed field in carbohydrate chemistry. Because of its wide distribution in plants, starch has always been a main item in the diet of man. As civilization progressed and expanded, the use of starches spread and new plant sources evolved.

The concept is widely accepted that there are two components in starch, namely amylose (a linear glucan) and amylopectin (a branched glucan). Successful methods of fractionation allowed each component to be studied separately. Amylose is a polymer of α - 1,4 glucopyranosyl units whereas amylopectin contains some α - 1,6 links in addition to α - 1,4. Such physical methods as x-ray diffraction and electronmicroscopy have been used successfully to study chain conformation of amylose. Because of branching which results in a complex configuration, those physical methods have not been very successful with amylopectin. Some carbohydrases, alpha-, beta-, and enzyme specific for α - 1,6 linkage have been used to advantage in the structure determination of amylopectin.

In the research reported in this thesis the structure of amylopectin in the granule was studied by a new technique, tritium atom bombardment. In this method a thin surface of the granule becomes labeled. The purpose of this research was to purify the labeled amylopectin, and to study the distribution of the activity in the molecule by use of glucoamylase. Since this enzyme degrades from the non-reducing end but will also hydrolyze α -1,6 links slowly, the specific activity of the glucose liberated should describe the distribution of activity in the molecule.

LITERATURE SURVEY

Structure of Amylopectin.

The ratio of amylose-amylopectin in many plant species has been studied (1). The amylopectin content of corn starch was generally in the range of 72 to 78%; the range for wheat 73 to 83%; sorghum 72 to 77%; oats 76 to 77% and rice 83 to 84%. Waxy maize, a mutant of corn, was found to produce starch with 100% of amylopectin.

Linkages.

The main types of linkages found by methylation in amylopectin are the α-D-(1$\rightarrow$4) and α-D-(1$\rightarrow$6) linkages. Although the α-D-(1$\rightarrow$3) linkage was reported in amylopectin from waxy maize, the presence of such a linkage has not been reported in other sources. Wolfrom and Thompson (2) isolated a small but definite amount of 3-O-α-D glucopyranosyl-D-glucose as its crystalline β-D-octaacetate from the acid hydrolyzate of waxy maize starch. The anomalous linkage in amylopectin was investigated by studying the periodate oxidation products (3). Amylopectin polyaldehyde having a degree of oxidation of 98.5% was reduced with sodium borohydride to the corresponding polyalcohol. After complete methylation of the polyalcohol, the resulting methylated derivative was hydrolyzed. The proportion of 2,6-di-O-methyl-D-glucose was very small (only 0.02%), and did not favor the presence of the so-called anomalous linkage of (1$\rightarrow$3) type.

Molecular Weight.

Early osmotic studies by Mayer (4) showed that the molecular weight of

amylopectin was in the range of 50,000 to 1,000,000 although it was emphasized that the value was probably low because of degradation during the dissociation process. Again Potter and Hassid (5) reported number average molecular weights in the range of $1x10^6$ to $6x10^6$ for amylopectins from different plant sources. However, Potter and Hassid found that the plot of osmotic pressure/concentration vs. concentration of amylopectin solution, was non-linear and thus the results are not reliable.

Number average molecular weights of amylopectin have also been obtained from chemical end-group assays. Using different reagent such as dinitrosalicylic acid to measure the reducing end groups, Meyer and co-worker (6) obtained values in the range of 160,000 to 260,000. Nussenbaum and Hassid (7) reported a value of 350,000 by the same method for amylopectin extracted from corn starch. A different result of $1x10^6$ was obtained by sedimentation and diffusion measurements (8).

The light-scattering method has also been used. This has the advantage that the sensitivity increases as the molecular weight increases. Zimm and Thurmond reported a weight average molecular weight of 250×10^6 for amylopectin (9). Witnauer, Senti and Stern (10) obtained a weight average molecular weight of 36×10^6 for potato amylopectin prepared by hot water dispersion and pentasol fractionation, and a value of 14×10^6 by autoclaving at 120°C. and nitrobenzene fractionation. The disagreement between the values obtained by light-scattering and osmotic pressure methods might be explained as a result of incomplete dissociation of granules which could cause extremely high values in weight average molecular weights as measured by the light scattering method (11). Another possible explanation was that the ratio of weight average molecular weight and number average molecular weight increases because of the heterogenity of the branch polymer (12). Erlander and Tobin (13)

recently reported values of 4×10^6 to 8×10^6 for waxy corn, amylopectin, by light-scattering.

Minor Component.

The first analysis of fatty substances in starch was made by Taylor and Nelson (14). They found that the granule structure had to be disrupted by hydrolysis before complete extraction of fatty substances could be achieved with non-polar fat solvents. Consequently, Schoch (15) proposed that the fatty substances in starch were attached by esterification to the carbohydrate. Later, Lehrman proposed that the fatty component was present as an adsorption complex with the carbohydrate (16). Schoch tested a series of solvents for their extraction efficiency (17). Polar solvents such as 80% methanol were more effective than non-polar solvents like chloroform or petroleum ether. The concept of an adsorption complex was supported by this finding. The concept that fatty substances might be held to amylose by "clathration" was implied by French and his co-worker (18).

It has been known for a long time that starch contains phosphorus. Schoch (17) extracted about 12% of the phosphorus of corn starch with methanol, and found that he could remove 24% of the phosphorus by electrodialysis from the unextracted and autoclaved sample. He suggested that most of the phosphorus was esterified to starch. Pasternak (19) identified D-glucose-6-phosphate from a partial acid hydrolyzate of the nexasaccharide which was isolated from potato starch after α-amylolysis. Thus the position of the phosphate group was located on carbon-6 of glucose. Lampitt and co-workers (20) confirmed that most of the phosphorus of potato starch was in the esterified phosphate form, bound to the amylopectin. A different observation was reported for wheat starch. Hodge and co-workers (21) suggested that

phosphorus might be present as phosphatides, bonded to the starch. This conclusion was reached for the amylose fraction; no direct evidence was found for amylopectin.

A study of the position of the phosphate group on the amylopectin molecule was carried out by Whelan (22). A limit dextrin containing all the α-D-(1$\rightarrow$6) linkages and about 20% of the original starch was obtained after α-amylolysis of waxy maize starch. The limit dextrin contained 0.011% of phosphorus as compared to 0.002% in the parent starch. Also, Radomski and Smith (23) found that the phosphorus content of amylopectin, amylose and the intermediate fraction recovered from the mother liquid after repeated amylose crystallization was 0.165, 0.008% and 0.083% respectively. The decreasing gradient of the phosphorus contents suggested a relationship between the branching linkages and phosphorus content.

Structural Aspects.

The accepted structure of amylopectin is the "bush" form, proposed by Meyer and Benfeld (24). Hirst and co-workers (25) showed that 2,3,4,6-tetra-O-methyl D-glucose, 2,3-di-O-methyl D glucose and 2,3,6-tri-methyl D-glucose were present in the hydrolysate of methylated amylopectin. The percentages of branching was calculated as follows:

$$\frac{\text{yield of tetramethyl glucose}}{\text{yield of trimethyl glucose}} \times 100\%$$

Periodate oxidation has long been used to determine the percentage of branching and number of glucose units in a segment (26). The number of tiers can also be calculated if the molecular weight is known. Stepwise degradation by enzymes has also been used to determine the structure of amylopectin. In a study of beta amylolysis of amylopectin, French (27) concluded

that the degradation ceases within one or two glucose units from the branching point. In addition to the exposed outer chains, there may be some chains in the inner part of the molecule that are structurally suitable for attack by enzyme but which are sterically protected and resistant to the enzyme.

Alpha-amylase is able to cleave branched molecules between the branch points (28). Singly branched oligosaccharides were isolated after extensive salivary amylolysis of waxy maize starch (29). It was shown that the blocking action of the branch point is not uniform in the three directions from the branch point. Doubly branched compounds were produced if the branch point occurs so close that amylase is unable to cleave between them. Manners (30) reported on the relationship between the alpha-amylolysis limit, and the degree of branching. He examined 30 different samples containing α-(1$\rightarrow$6) linkages of which the percentages of branching were known. The P.M. value which refers to the maximum apparent percentage conversions into maltose was linearly related to the percentage of 1$\rightarrow$6 linkage.

The structure of α-amylase macrodextrin was examined by Karol and his co-worker (31). From a mixture of dextrin prepared by α-amylolytic degradation of potato starch, two homogeneous macrodextrin fractions were separated on a sephadex G-25 column and were characterized by the degree of branching. In the macrodextrin, chains were on an average, three to four glucose units apart, whereas in native amylopectin they averaged five to nine glucose units apart.

The fine structure of amylopectin was investigated by Lee and co-worker (32). An extracellular bacterial enzyme, pullulanase, specific for the hydrolysis of the α-D-(1$\rightarrow$6) linkage was used to debranch the amylopectin. Fractionation of the debranched polysaccharides was performed on a sephadex G-50 column. A convenient method for the determination of chain

length was carried out by measurement of the wavelength of peak absorption of the iodine-stained fraction as described by Bailey and Whelan (33). There is a linear relationship in a plot of the wavelength of peak absorption vs. chain length for amylose type chains of DP 20-60. Waxy maize starch and beta-amylase limit dextrin of waxy maize starch were examined in this way. To reconcile the distribution patterns from amylopectin and beta-dextrin it was concluded that there were few unit chains in amylopectin with length greater than 80 glucose units. This is consistent with a multiply-branched amylopectin with a molecular weight of about one million.

Glucoamylase

Sources.

Glucoamylases (α-D-(1$\rightarrow$4)-glucan glucohydrolases) capable of hydrolyzing starch directly to D-glucose, were found in several species of fungi of the Aspergillus (34) and Rhizopus groups (35) and in certain bacteria and yeasts, Clostridium acetobutylicum (36) and Saccharomyces diastaticus (37). Chromatography on a DEAE-cellulose column provided a way to purify the enzyme from other contaminating carbohydrases. Glucoamylases have also been detected in animal tissues particularly in the liver (38). Other names have been employed for this type of enzyme such as amyloglucosidase and gamma-amylase.

Composition.

Glucoamylase from Aspergillus niger was found to contain significant amounts of carbohydrates which were attached to the polypeptide chains of the enzyme (39). Carbohydrate residues found were D-mannose, D-glucosamine, D-glucose and D-galactose. It was postulated that the carbohydrate units were esterified to the carboxyl groups of the glutamic and aspartic acid of poly-

peptide chain. Pazur and Okada (40) purified glucoamylasls from Rhizopus delemar by successive gel filtration on sephadex G-50, Duolite A-2 and Amberlite XE-64 resin. The homogenous enzyme obtained in this way was shown to be a glycoprotein containing D-mannose and 2-amino, 2-deoxy-D-glucose.

Substrate Specificity.

Pure glucoamylase hydrolyzed amylopectin (both α-D-(1$\rightarrow$4) and α-D-(1$\rightarrow$6) links), amylose (α-D-(1$\rightarrow$4) links), malto-oligosaccharide (α-D-(1$\rightarrow$4) type), isomaltose (α-D-(1$\rightarrow$6)) and nigerose (α-D-(1$\rightarrow$3)) completely to D-glucose. Measurements of initial rate of hydrolysis of maltose, nigerose and isomaltose (41) were 30:3:1.

Since isomaltose, maltose and nigerose acted as competitive inhibitors to each other, it was concluded that these three types of linkages were hydrolyzed at the active site of the enzyme (42). The hydrolysis of amylopectin by glucoamylases begins at the non-reducing ends (43) and continues down the chain removing single D-glucose units. When the α-D-(1$\rightarrow$6) linkage is reached, it is hydrolyzed also, but at a slower rate, thus permitting the enzyme to bypass this barrier. A multi-chain mechanism on malto-oligosaccharides was claimed by Pazur and Okada for glucoamylase from Rhizopus delemar (40).

Optimum pH and temperature have been reported for glucoamylase from a number of sources. Lo and co-workers (44) reported an optimum pH of 4.5 to 5.0 and optimum temperature 50 to 55° for glucoamylase from Rhizopus. Helena Ebertora reported an optimum pH of 4.5 and an optimum temperature of 40 to 50° for glucoamylase from E. Cansularis (45). King reported an optimum pH of 4.0 to 4.5 for glucoamylase from Coninphora cerebella (46). An unusual

tolerance to high temperature is a characteristic of this enzyme.

The activation energy of the hydrolysis was measured by Helena Ebertora (46) to be 7200 cal at a temperature of 20 to 40°. She reported that the reaction rate decreased with decreasing molecular weight. Substrates which contained α-D-(1$\rightarrow$6) glucosidic bonds were hydrolyzed slower (factor of 50) than compounds containing α-D-(1$\rightarrow$4) bonds having the same length of glucosidic chain.

A kinetic study of glucoamylases hydrolysis was made by Keitaro Hiromi and co-workers (47). Crystalline glucoamylase isolated from Rhizopus delemar had a Km for isomaltose, panose and maltose of 2.5×10^{-2}M, 6.9×10^{-3}M and 1.1×10^{-3}M respectively at 25° and pH of 5.1. The pK values of the initial ionizable groups of the free enzyme involved in the hydrolysis were 2.85 and 5.85.

Mercuric salts was found to inhibit the enzymic reaction of glucoamylase completely, but other ions tested had little effect on the activity, at the concentration of ion used (0.005M) (46). The initial ratio of glucose production from amylopectin, amylose and maltose were in the proportion of 40:10:1.

Side Reaction.

The products of the reaction of D-glucose with a crude glucoamylase from Aspergillus niger were examined by Watanabe Toshiyuki and co-workers (48). A 40% D-glucose solution was incubated with a 0.68% of crude glucoamylase at 55° for 72 to 96 hours, followed by inactivation of the enzyme at 80°. The reaction products were analyzed by paper chromatography, paper ionophoresis, carbon-celite and Magnesol-celite column chromatography. Kojibiose, nigerose, maltose, panitol, laminaribiose, isomaltose and isomaltotriose were demonstrated. Prolonged and extensive hydrolysis could caused D-glucose reversion products.

METHODS AND MATERIALS

Determination of Total Carbohydrate (49).

One ml of 5% (by volume) aqueous phenol solution was added to one ml of carbohydrate solution and mixed well. A blank was prepared with one ml of distilled water instead of carbohydrate solution. Five ml of 96% sulfuric acid was added quickly with mixing to provide a good heat distribution. After ten minutes, the tube was placed in a water bath at 25 to 30°C for 20 minutes. The yellow orange color was stable for several hours. Absorbance was measured at 490 mu in a Bausch and Lomb spectronic 20 photoelectric colorimeter. A plot of absorbance vs. amount of carbohydrate was linear with small variation, to at least 80 ug with a value of 0.0099 O.D. units/ug carbohydrate in a one cm cuvette.

Radioactivity Measurement.

The activity of tritium labeled starch was measured with a Beckman LS-200B Scintillation Counter (a soft beta counting spectrometer). Two kinds of scintillator were used: 100g of naphthalene, 5g of 2.5-diphenyl oxazole (Packard Instrument Company, Inc.) per liter of spectroquality dioxane (Matheson Coleman and Bell Co.); 5g of 2.5-diphenyl oxazole per liter of toluene. One ml of carbohydrate solution was added to 15 ml of dioxane counting solution. A different procedure was used for the toluene scintillator. The sample was dried on a circular glass fiber disc of diameter 2.4 cm (Reeve Angel, Clifton, N. J.) then immersed in 5 ml of counting solution. For all measurements, the counting efficiency was about 43% for

the dioxane and 55% for the glass fiber disc in toluene when checked with the tritium standard.

Self-absorption may cause error in the determination of the radioactivity of a low energy beta particle emitter such as tritium. Various amounts of inactive starch mixed with about 25 ug of active starch were counted on the glass fiber discs. The measurements were made in 5 ml of toluene scintillator. No self-absorption was observed from a plot of radioactivity vs. amount of starch over the range of 25 mg to 1420 mg.

Preparation and Fractionation of Tritiated Starch.

The method of Moser, Nordin and Senne (50) was followed to label the sample by exposure to energetic tritium atoms. Three hundred mg of waxy maize starch was suspended in 10 ml of acetone and coated on the reaction vessel (14 x 5 cm) by evaporation of acetone with a vacuum drier while the vessel was continuously rotated. During labeling the reaction vessel was maintained at the temperature of liquid nitrogen. The filament at a temperature of 2100°K was centered in the reaction vessel. Tritium gas was introduced through a palladium thimble at a controlled rate of a few $\frac{mg}{min}$. The pressure in the reaction vessel was 0.3×10^{-3} torr (one torr = one mm Hg) and the reaction time was 2½ minutes. After the reaction, the vessel was warmed up to room temperature and the tritium gas was pumped off.

The labeled starch was purified as follows: It was first washed with 50 ml of methanol to remove exchangeable tritium and certain side reaction products. It was then dissolved in dimethyl sulfoxide (62.5 $\frac{mg}{ml}$ of DMSO) followed by the addition of water (final solution) 1% and precipitated by the addition of either one volume of acetone or two volume of ethanol. After precipitation it was passed through a DEAE-Sephadex A-50 anion exchange column (Pharmacia Fine Chemical Inc.). The method of Schmidt (51) was used to

activate the exchanger. DEAE-Sephadex in powder form was first soaked in 1.0M NaCl solution overnight and then washed in distilled water repeatedly for 24 hours. Before preparation of the column (70 x 1.8 cm) the exchanger was kept in 0.01 M ammonium acetate buffer at pH=8. Aqueous radioactive amylopectin solution was injected into the column and eluted with 0.01M ammonium acetate buffer (pH=8). Gradient elution with 0.2M NaCl, 0.5 M NaCl, 1.0M NaCl, 0.01 N HCl and 1.0 M NaCl, 0.1N HCl and 1.0 NaCl was carried out. Fractions of five ml were collected with an automatic fraction collector.

Iodine Absorption Spectra.

Radioactive amylopectin was characterized by determining the iodine absorption spectra as described by McCready and Hassid (52). To 0.5 mg of amylopectin in one ml of distilled water, 0.5 ml of iodine solution (20 mg of KI and 2 mg of iodine per ml) was added. It was made up to 50 ml with distilled water and allowed to stand for 20 minutes at room temperature. A blank was prepared in the same manner except with one ml of water instead of amylopectin solution. The absorbance was measured against the reagent blank in a Backman DU -2 spectrophotometer over the range of 510-580 mu.

Enzymic Degradation of Labeled Amylopectin.

Highly purified glucoamylase from Aspergillus niger was obtained from Dr. Lineback, University of Nebraska, Lincoln, Nebraska. About 15 mg. of DEAE-Sephadex purified amylopectin was dissolved in 3 ml of water and 6.7 ml of 0.05 M citrate buffer was added to keep the pH around 4.5. To get data at low percentages of hydrolysis as well as close to 100% hydrolysis, a total amount of 3 ml of glucoamylase (0.84 mg protein per ml was added at the following times: 0.3 ml at zero time, 0.7 ml at 15 minutes, 1.0 ml at 24 hours, and another 1.0 ml at 36 hours. Samples of hydrolyzate were

withdrawn from the reaction vessel at various time intervals throughout the 41 hours period. The amount of hydrolyzate withdrawn was varied in proportion to the degree of hydrolysis to ensure that a sufficient amount of glucose was present for analysis.

Charcoal-celite column chromatography was used to separate glucose from untreated amylopectin (53). Equal parts by weight of activated charcoal (Norit-A, Fisher Scientific Co.) and celite (Warner-Chilcott Laboratories) were mixed. The water was added to make a slurry. About 3 ml of the slurry was added to a small column (15 x 1.2 cm) containing a small pad of cotton and glass wool. The column was washed with distilled water and suction was used to pack the column tightly. Column background was checked to make sure no contaminants of carbohydrate and radioactive material were present. The glucoamylase hydrolyzate was then applied to the column and about 70 ml of effluent was collected. In a trial experiment 100% recovery of glucose was found when the column was washed with water. The effluent was evaporated to dryness under vacuum and 1.5 ml of water was added to redissolve the glucose. The recovery of carbohydrate was found to be 100%. The experimental procedure was kept constant so as to reduce the possibility of variance due to the loss of labeled tritium. Under the condition used in this experiment nearly 100% of exchangeable tritium was removed.

Strip Counting of Paper Chromatograms.

To examine the purity of glucose from charcoal-celite column, the concentrated effluent was spotted 3 cm from the edge of a 25 x 20 cm unwashed Whatman No. 1 chromatographic paper. A mixture of glucose and maltose was spotted beside it as a reference. The chromatogram was developed in a solvent system of 1-butanol, ethanol and water (6:4:3 by volume) for about 12

hours. The developed chromatograms were cut into one cm strips parallel to the base line of the chromatogram. Each strip was then cut into two parts and put directly in 5 ml of toluene and 2,5-diphenyl oxazole scintillator. The radioactivity was measured with a Beckman LS-200B Scintillator Counter.

RESULTS AND DISCUSSION

Purification of Radioactive Amylopectin

Purification of tritiated amylopectin was carried out in two steps, (1) dissolving in dimethyl sulfoxide followed by precipitation with either acetone (50% by volume) or ethanol (68% by volume); (2) by passing through a DEAE-Sephadex column. Tritiated amylopectin from potato and waxy maize starch were partially purified by precipitation with acetone or ethanol. This procedure provided a means of eliminating the low molecular weight dextrins which are not precipitated under these condition (54). Chromatography on DEAE-Sephadex separated the amylopectin from potato starch into three peaks (Fig. 1). Although DEAE-Sephadex is capable of separating according to molecular size, the sizing effect appears in the first few fractions only. Thus peak I, II and III in Fig. I probably differ in the number of charges on the amylopectin molecule. Phosphate groups attached near the branch point of amylopectin (22) could be the cause of this fractionation. Thus the peaks in Fig. 1 may differ from each other mainly in phosphate content rather than in molecular size. Since DEAE-Sephadex is an anion exchanger, peak III should contain more phosphate groups than peak I and peak II. DEAE-Sephadex column chromatography of waxy maize starch (Fig. 2) gave essentially only one peak close to the void volume, suggesting a low phosphate content. Reports in the literature appear to confirm this conclusion (55). A value of 0.09% was reported for methanol extracted potato amylopectin and 0.007% for waxy maize starch.

In Fig. 1, the pH value of the elutant was decreased from 8 to 2 in the first one hundred fractions. The gradual increase of normality of HCl Solution leads to a change of pH from 2 to 1 and, then 1 to 0.48. Acid hydrolysis of the exchanger itself was apparently occurring in the last samples collected, because of the low pH. Thus more than 100% carbohydrate was eventually eluted. Tubes after 138 were therefore eliminated because close to 100% of the activity had been collected at this point. The low background at tube 140 suggests that peak III was not greatly contaminated with extraneous carbohydrate from the column.

The fractionated potato amylopectin peaks were characterized by their iodine absorption spectra. Iodine in KCl solution absorbs weakly with a maximum near 450 mu. Upon the addition of small amount of amylose this peak shifts to longer wavelengths, giving a blue color and an intense absorption peak with the maximum near 600 mu. Amylopectin exhibits a spectrum with maximum absorption in the neighborhood of 550 mu. The maximum absorption may vary somewhat. It depends on the degree of polymerization of amylose and the length of outer chain in amylopectin. However, nearly all samples of amylose absorb near 600 mu with a much higher absorption coefficient than amylopectin. The use of iodine absorption spectra provides an easy way to examine for possible contamination of amylose in the amylopectin solution. With a small amount of amylose present the absorption peak shifts from the neighborhood of 550 mu to a longer wavelength and also gives more intense absorption. Peak I, peak II and peak III in Fig. 1 were examined by iodine absorption spectra as described earlier. The results are shown in table 1. The maximum absorption of peak I, peak II and peak III were 545, 558 and 545 mu respectively. The absorption peaks are typical of amylopectin. The higher

value for peak III is apparently not due to amylose contamination because the entire spectrum was shifted and the absorbance values were not increased. They were actually lowered. This becomes apparent when the data is plotted.

All three peaks were hydrolyzed with glucoamylase. The glucose produced was passed through a charcoal-celite column. Peak I and peak II were treated with 1.0 ml of glucoamylase whereas peak III was treated with 2.25 ml of this enzyme. The enzymic degradation was allowed to proceed for three days. After passing through the charcoal celite column the amount of glucose and the radioactivity in the effluent were measured. The percentages of hydrolysis were 84%, 69.5%, 100% and the recovery of radioactivity were 33.3%, 43.5%, 38% for peak I, peak II and peak III respectively.

Close inspection of the data and of Fig. 1 reveals some differences which should be confirmed by further work. Peak I (Fig. 1) had a higher specific activity than peak II and peak III. Recovery of activity in glucose after glucoamylase treatment however varied only slightly between peaks. This was judged to be insignificant. The remainder of the research was therefore conducted with waxy maize starch since it was more easily purified than amylopectin from corn or potato starch.

Enzymic Degradation of Labeled Waxy Maize Starch.

Waxy maize starch purified by DEAE-Sephadex column chromatography was used to study the glucoamylase degradation pattern. The results are shown in Fig. 3 and table 2. The specific activity declined from 0% to 60% of hydrolysis and then remained constant. Total radioactivity of glucose increased almost linearly after about 30% of hydrolysis. A slight concentration of activity in outer branches is indicated by this data, confirming the observation of Rao (56). The monosaccharide produced in the enzymic degradation

of waxy maize starch was examined by paper chromatography and glucose was found to be the only product (table 3).

Strip Counting of Paper Chromatogram.

To examine the radioactive purity of glucose produced in glucoamylase degradation, fractionated amylopectin peak I, peak II, peak III in Fig. 1 and the single peak in Fig. 2 were hydrolyzed to glucose by glucoamylase. Strip counting was carried out after charcoal-celite column separation and paper chromatography. In all cases, only one major peak was found corresponding to glucose in Rf. A method of strip counting of paper chromatograms was described by Yadagiri (57). In this method, the sugar on the paper chromatogram strip was eluted with water as described by Dimler et al (58), and the radioactivity of elutant measured. A simpler method was used in this study in which the paper chromatogram strip was immersed directly into the toluene scintillator. Although it is qualitative rather than quantitative, it was quite satisfactory for scanning. The efficiency of the radioactivity measurement was estimated to be 6%.

Distribution of Tritium and Structure of Waxy Maize Starch Granules.

Several postulated structures for starch granules should now be examined in the light of this data: (1) The amylopectin molecules are arranged antiparallel (59) but perpendicular to the surface; (2) The molecules are folded in such a way than anti-parallel arrangement of chains occur. The molecules may be antiparallel or parallel but again chains are perpendicular to the surface (60); (3) Finally, the oldest theory first advanced by Meyer (61) and supported by Rao and Yadagiri (56, 57) is that molecules are parallel with non-reducing ends at the surface and are a result of biosynthesis at the surface.

The results shown here are best explained by the third postulate although presumably it may be possible to fold chains in such a way that non-reducing ends are at the surface. Postulate (1) seems to be entirely excluded by the data presented here.

Finally, the data shows that labeling occurs beyond the branch point in a uniform manner. The only reasonable explanation is that the surface is quite amorphous and that the tritium atoms penetrate deeper than one molecular layer. In postulate (1) we might expect an upturn of activity as hydrolysis by glucoamylase approached 100%. The data on the contrary suggests that it is uniformly distributed beyond the branch point.

A fourth possibility may be postulated for the results shown here, a random arrangement. Concentration of activity in outer branches could occur because of the structure of the molecule itself, i.e. branches may be clustered around the interior of the molecule in such a way that penetration by tritium atoms is reduced even though the structure is random. The only way to check this point is through a series of experiments with pregelatinized starch. What kind of degradation pattern would be shown by the reaction of glucoamylase on tritium labeled gelatinized starch? It is proposed that an extensive survey should be made on the effect of gelatinization under various conditions. Also the effect of certain agents which are thought to complex with outer branches should be tried. Only by the use of such blanks could one properly subtract out the internal effect, i.e. the effect due to the structure of the molecule itself.

EXPLANATION OF FIGURE 1

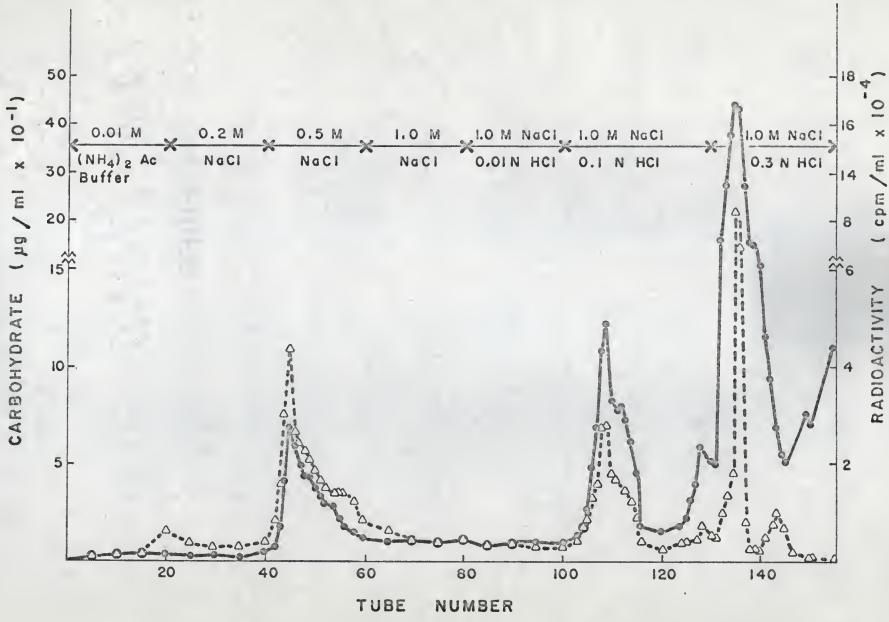
Fractionation of Tritiated Potato Amylopectin on DEAE- Sephadex Anion Exchanger

Recovery: Carbohydrate - 94.7% (fractions after tube 138 were neglected due to the decomposition of exchange itself)

Radioactivity - 96.6%

△ Radioactivity

● Carbohydrate


0.01 M
(NH4)2 Ac
Buffer
0.2 M
NaCl
0.5 M
NaCl
1.0 M
NaCl
1.0 M NaCl
0.01 N HCl
1.0 M NaCl
0.1 N HCl
1.0 M NaCl
0.3 N HCl
CARBOHYDRATE (µg / ml x 10-1)
RADIOACTIVITY (cpm / ml x 10-4)
TUBE NUMBER
50
40
30
20
15
10
5
18
16
14
8
6
4
2
20
40
60
80
100
120
140

EXPLANATION OF FIGURE 2

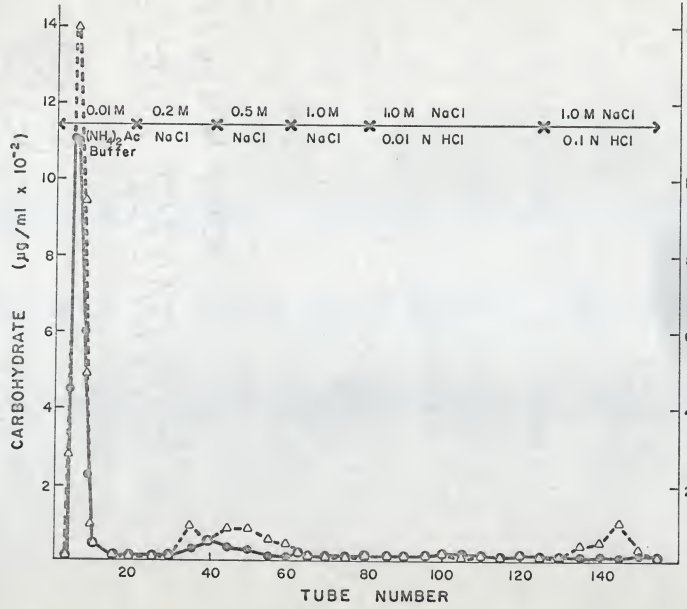
Purification of Tritiated Waxy Maize Starch on DEAE-Sephadex Anion Exchanger

Recovery: Carbohydrate - 78.8%

Radioactivity - 74.5%

● Carbohydrate

△ Radioactivity


0.01 M
(NH4)2Ac
Buffer
0.2 M
NaCl
0.5 M
NaCl
1.0 M
NaCl
1.0 M NaCl
0.01 N HCl
1.0 M NaCl
0.1 N HCl
CARBOHYDRATE (μg/ml x 10-2)
RADIOACTIVITY (cpm/ml x 10-4)
TUBE NUMBER
2
4
6
8
10
12
14
20
40
60
80
100
120
140

EXPLANATION OF FIGURE 3

Glucoamylase Degradation Pattern of Waxy Maize Starch Purified on a DEAE-Sephadex Column

Recovery in effluent from the charcoal-celite column at 100% conversion

Carbohydrate - 100%

Radioactivity - 30%

o Radioactivity of glucose

Δ Specific activity of glucose

SPECIFIC ACTIVITY OF GLUCOSE (cpm/µg)
RADIOACTIVITY OF GLUCOSE (cpm ml x 10^{-3})
PERCENTAGE CONVERSION TO GLUCOSE
20
10
10
5
20
40
60
80
100

TABLE 1

IODINE ABSORPTION OF PEAK I, II, and III OF FIG. 1

Wavelength mu	Absorbance Peak I	Peak II	Peak III
510	0.140	0.100	0.113
520	0.150	0.112	0.120
530	0.155	0.117	0.124
540	0.157	0.126	0.127
550	0.157	0.128	0.127
560	0.155	0.130	0.126
570	0.145	0.123	0.118
580	0.138	0.117	0.112

Wavelength of maximum absorbancy were:

Peak I: 545 mu, Peak II: 558 mu, Peak III: 545 mu

TABLE 2

HYDROLYSIS BY GLUCOAMYLASE OF PURIFIED
TRITIATED AMYLOPECTIN (1180 ug/ml, 4200 cpm/ml)

Time	Glucose converted[1] Carbohydrate (ug/ml of digest)	Radioactivity (cpm/ml of digest	Percentage hydrolysis	Specific Activity (cpm/ug)
0.5 min	22	400	1.87	18.2
2.0 min	55	1120	4.66	20.2
5.0 min	155	2400	13.1	15.5
15.0 min	355	4800	30.5	13.5
30.0 min	628	6300	53.2	10.0
1.0 hr.	809	9100	68.6	11.2
7.0 hr.	978	10800	82.8	11.0
24.0 hr.	1110	13300	94.0	12.0
31.0 hr.	1196	12800	101.2	10.7
36.0 hr.	1176	12100	99.6	10.3
41.0 hr.	1178	12000	99.8	10.2

1. Measurements were made on the charcoal-celite column effluent (70 ml). The following amounts of digest were applied to the column

Sample	1	- 3	1 ml
	4	- 11	0.5 ml

TABLE 3

STRIP COUNTING OF PAPER CHROMATOGRAM

Distance from origin (cm)	Radioactivity (cpm) A	B	C	D
1	80	10	0	16
2	30	0	2	1
3	20	0	5	0
4	18	0	1	6
5	4	0	0	6
6	3	0	2	0
7	3	2	0	2
8	80	68	15	9
9	395	485	152	51
10	25	70	78	140
11	0	0	0	52
12	00	6	2	8
13	0	3	5	6
14	0	0	0	0
15	0	6	0	0

A. Peak I of Fig. 1, 84% hydrolysis and 33.3% of activity recovered in glucose (based on measurements of charcoal-celite column effluent)

B. Peak II of Fig. 1, 69.5% - - - - - - - - 43.5%

C. Peak III of Fig. 1, 100% - - - - - - - - 38.0%

D. Peak I of Fig. 2, 100% - - - - - - - - 30.0%

SUMMARY

1. Fractionation of tritiated potato amylopectin was carried out in two steps. (1) Partial fractionation by precipitation either with 66% ethanol or 50% acetone. (2) Passing through a DEAE-Sephadex column. Samples after these treatments could be fractionated into three peaks by DEAE-Sephadex column chromatography. It was postulated that fractionation was attained because of the difference in phosphate content.

2. Tritiated waxy maize starch purified in the same manner as 1. above yielded a single peak by DEAE-Sephadex column chromatography.

3. The distribution of activity in the labeled starch was studied by examining the degradation pattern produced by glucoamylase. Specific activity of glucose decreased as the percentage of hydrolysis increased and remained constant after 60% of hydrolysis. The total activity of glucose increased almost linearly after 30% of hydrolysis.

4. The experimental results supports the Meyer theory of starch granule structure in which the molecules are postulated to be arranged parallel with non-reducing ends at the surface.

5. The enzymic degradation pattern supports the concept that labeling occurred beyond the branch point in a uniform manner.

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PURIFICATION AND CHARACTERIZATION

OF

TRITIUM LABELED STARCH

by

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AN ABSTRACT OF A THESIS

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1969

ABSTRACT

Fractionation of tritiated amylopectin was carried out by precipitation with 68% ethanol or 50% acetone followed by passing through DEAE-Sephadex column. Three peaks which did not differ very significantly in recovery of activity in glucose were found for potato amylopectin. A single peak was found for waxy maize starch.

Glucoamylase was used to study the distribution of labeling in waxy maize starch purified by DEAE-Sephadex. It revealed that waxy maize starch molecules are laid down in the granule with non-reducing ends at the surface. Labeling occurs beyond the branch point in a uniform manner.